MULTIPLEX SOLUTION GENOTYPING WITHOUT PROBES OF THE FACTOR V LEIDEN (G1691A), PROTHROMBIN (G20210A), AND MTHFR (C677T AND A1298C) MUTATIONS IN ONE TUBE BY SINGLE COLOR HIGH-RESOLUTION MELTING ANALYSIS.

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Background: Venous and arterial thrombotic diseases are serious health concerns with well established links to the Factor V Leiden (G1691A), Prothrombin (G20210A), MTHFR (C677T) and MTHFR (A1298C) mutations. The likelihood of developing disease is increased with the presence of mutations in multiple clotting factor genes.

Methods: Four amplicons with non-overlapping melting temperature windows were created by varying the amplicon lengths from 40 base pairs to 120 base pairs and by using primers with G/C rich tails. Genotyping of each locus in the same closed-tube was based on the shape and position of the high-resolution melting transition. Heteroduplex melting analysis with LCGreen eliminated any need for probes.

Results: The Tm windows for each gene were separated by at least 1°C with the wild type melting temperatures of 76.0°C for Factor V Leiden, 88.5°C for Prothrombin, 84.5°C for MTHFR C677, and 80.5°C for MTHFR A1298. Within each window, the wild type, mutant, and heterozygous genotypes for each mutation, were resolvable. Concordant results between conventional LightCycler analysis and high-resolution melting were obtained on 120 blinded clinical samples.

Conclusions: Genotyping by amplicon melting is a rapid, closed-tube method for genotyping without probes that can be multiplexed. The method was successfully applied to the four most common clotting factor mutations in a single tube. The

simplicity of the method allows rapid development of low-cost assays and does not require any arrays or complex instrumentation.